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A mammalian protein targeted by G1-arresting rapamycin-receptor complex

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THE structurally related natural products rapamycin and FK506 bind to the same intracellular receptor, FKBP12, yet the resulting

complexes interfere with distinct signalling pathways^{1,2}. FKBP12-rapamycin inhibits progression through the G1 phase of the cell cycle in osteosarcoma³, liver^{4,5} and T cells^{6,7} as well as in yeast⁸, and interferes with mitogenic signalling pathways that are involved in G1 progression^{9,10}, namely with activation of the protein p70^{SEK} (refs 5, 11-13) and cyclin-dependent kinases^{3,14-16}. Here we isolate a mammalian FKBP-rapamycin-associated protein (FRAP) whose binding to structural variants of rapamycin complexed to FKBP12 correlates with the ability of these ligands to inhibit cell-cycle progression. Peptide sequences from purified bovine FRAP were used to isolate a human cDNA clone that is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products from *Saccharomyces cerevisiae*^{8,17,18}. Although it has not been previously demonstrated that either of the *DRR/TOR* gene products can bind the FKBP-rapamycin complex directly^{17,19}, these yeast genes have been genetically linked to a rapamycin-sensitive pathway and are thought to encode lipid kinases¹⁷⁻²⁰.

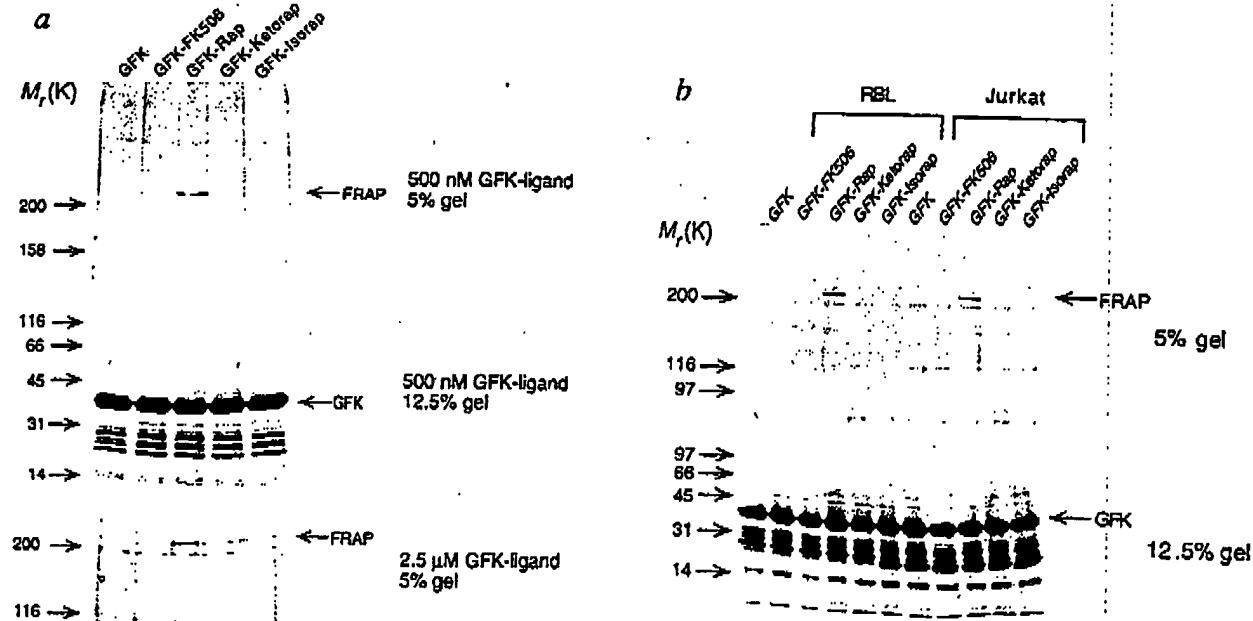


FIG. 1. Identification of FRAP protein in three mammalian cell lines. *a*, GFK alone or individual GFK-ligand complexes were added to MG-63 cell lysates (2×10^7 cells per condition) to a final concentration of either 500 nM or 2.5 μ M and the mixtures incubated for 10 min at 4 °C. Fusion protein complexes were recovered by glutathione-affinity chromatography, and the proteins detected by silver staining after 5% SDS-PAGE. Because of compression, FRAP is not resolved by 12.5% SDS-PAGE, so both 5% and 12.5% gels are shown. The amount of FRAP that was retained by affinity chromatography saturated at concentrations of GFK-Rap greater than 500 nM in these experiments and in others using concentrations of GFK-Rap ranging from 100 nM to 5 μ M (data not shown). *b*, GFK alone or individual GFK-ligand complexes were added to a final concentration of 500 nM to lysates prepared from either 2×10^8 Jurkat T lymphocytes or 10^6 rat basophilic leukaemia (RBL) cells per condition. Lysates were treated as in *a*. FKBP12, but not FKBP13 or FKBP25 (ref. 23) is able to mediate the actions of rapamycin in *S.*

cerevisiae. In addition, we found that YFK188 (ref. 24), an FKBP12 null strain, could be complemented with GFK (P. K. Martin, B. Gladstone, G. Weiss, D. T. Hung, S.L.S., in preparation). Thus the GST appendage of the fusion protein does not preclude binding of the biologically relevant target to the GFK-rapamycin complex in yeast.

METHODS. MG-63, Jurkat and RBL cells were grown in media containing 10% FBS and lysed at 4 °C in PINT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μ M NaVO₄, 25 mM 2-glycerophosphate, 0.2 mM PMSF, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin A and 2 mM ODT) containing 0.5% Triton X-100. Lysates were clarified by centrifugation at 25,000g, and the Triton X-100 in the supernatant was diluted to 0.33% by adding 0.5 vol PINT buffer. GFK prebound to stoichiometric quantities of FK506, keto-isopod or unmodified rapamycin was added to lysates as described. Each condition was then passed through a 250- μ l glutathione-Sepharose column, which was washed with PINT buffer containing 0.5 M NaCl and 0.3% Triton X-100.

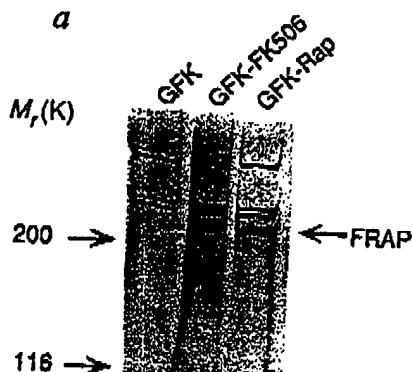
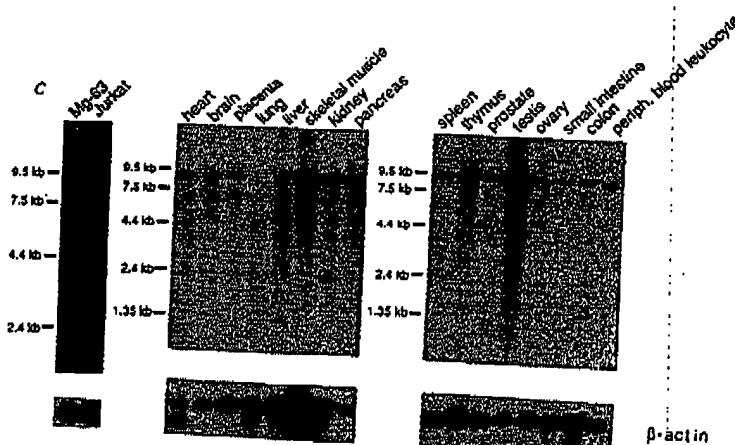


FIG. 2 Purification of FRAP from bovine brain and cDNA cloning of human FRAP. **a**, Fivefold-enriched bovine FRAP (S-column eluate; see below) was conditioned with [100 nM], glutathione-S-transferase-fusion protein (GFK), GFK-FK506 or GFK-Rap. Complexes were detected as described in Fig. 1 legend. We also found FRAP in bovine liver and thymus. **b**, Predicted translational product of the human FRAP cDNA clone. Bovine FRAP peptide sequences aligned to human FRAP are indicated by underlined segments. In the reading frame shown translational stop codons were not encountered upstream of the initiating methionine. **c**, Northern blot analysis of human tissue, Jurkat T cell and MG-63 cell poly (A)⁺ RNA. The Jurkat/MG-63 and multiple tissue Northern blots (Clontech) were hybridized with ³²P-labelled probes derived from the 182 bp PCR fragment and the 5.5 kb clone (text), respectively. Hybridization to human β -actin probe is shown as an internal control for loading.

METHODS. Bovine FRAP was purified by grinding 900 g of bovine brain in blender with 1 litre of PIP (0.3% Triton X-100, 50 mM sodium phosphate, pH 7.2, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μ M Na₃VO₄, 25 mM 2-glycerophosphate, 1 mM PMSF, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin A, 1 mM benzamidine and 2 mM DTT). The homogenate was centrifuged at 25,000g and the supernatant (20 g total protein) was loaded onto a 1 litre S-Sepharose (Pharmacia) column. The column was then washed with PIP and eluted with PINP (PIP with 1M NaCl). GFK-rapamycin was added to the pooled eluate to a final concentration of 100 nM and recovered by glutathione-affinity chromatography. FRAP was resolved by SDS-PAGE and transferred to PVDF. Following digestion with trypsin or endoproteinase Lys-C (Boehringer Mannheim) bFRAP peptides were microsequenced²³. The Jurkat T cell cDNA library (Stratagene) was constructed through random and oligo dT priming of cytoplasmic oligo dT purified RNA (ref. 25). cDNA screening, Jurkat and MG-63 RNA isolation and northern blotting and were performed by procedures similar to those previously described²³. A 182 bp fragment was amplified from a human brain stem library (Stratagene) and labelled by incorporation of ³²P-dCTP in the course of reamplification by PCR. The sequences were analysed using BLAST (ref. 26) and the University of Wisconsin GCG (ref. 27) software. The human FRAP cDNA sequence has been submitted to Genbank.

We used two structural variants of rapamycin, 16-keto-rapamycin (S. D. Meyer and S.L.S., manuscript in preparation) and 25,26-iso-rapamycin²¹, to identify any biologically relevant targets of the FKBP-rapamycin complex. Both variants bind tightly to human FKBP12, as shown by their ability to inhibit rotamase activity of the recombinant protein (K_i values were 0.2 nM for rapamycin⁶, 2 nM for keto-rapamycin, and 0.1 nM for iso-rapamycin). But the variants are about two orders of magnitude less potent than rapamycin in preventing the progression through G1 of MG-63 human osteosarcoma cells. The values of IC₅₀ (half-maximal inhibitory concentration) estimated from dose-response curves are 0.1 nM, 7.5 nM and 50 nM for rapamycin, keto- and iso-rapamycin, respectively. Thus the complexes of iso- and keto-rapamycin with FKBP12 should bind to

1	MIGCTGPAAT TAATISSSSVS VLQQFASGLK SRNEETRAKA AKELQHVVTH	50
51	ELRENSQES TREYQDNNH TEFIVQSSSDA NERKGIGLAI ASLIGVVEGN	300
101	ATRICRPFANY LRNLPSNDP VVNMDSKAI GRALAGDTF TAEYVEFEVK	250
151	RALEMICADR NECRHHAALV VLRLAISPV TFFFQVQPF FDNIFVAVND	200
201	PKQAZREGAV AALRACLHQK TOREPKHEQK PQWYRQNTFEE AEKGFDTELÄ	250
251	KEKGUNDRDR IRGALLILNE LVRISSSMEGE RLREEMEET QOOLVHDYRC	300
301	KOLMGECTKPK RWTPTPESQ AVDQPSQSL VGLGYSSHQ GLMGFTSPS	350
351	PAKSTLVESR CCRDNEEKF DCQVCQW/LKC RNSKNSLTON TILNLLP	400
401	AFRPSAFTDT QYQDTHNNH LSCVXKEKER TAAFPQALCLL SVAVRSEPKV	450
451	YLPRVLDIYR AALPPKDFAH KROKAMQVDR TVFTCISHAL RAMGPQIQOD	500
501	IKELLEPHMLA VGLSPALTAW LYDLSRQIPQ LKKQZQDGL KHLSLVLMHK	550
551	PLRHPGMPIG LAHQQLASPLG TTPEASDVG SITLARLTG SPEFEHGSLT	600
601	QFVRKCADHF LNSEHKEIRL EAARTCSRL TPSJHLTSGH AHVYVQTAQ	650
651	VVAQVLSSKL VVQITDPOPE IRYCVLSDA ERFDAHLAQI ENLQALFVAL	700
701	NDQVFEIRL AIXTYGRLLS NAPAFVMPFL RKLXLIQILTE LEHSGCIGRIZ	750
751	EQSARMILGHI VSNAPIRLRD YMEPIKALI LKLKOPDOPP KPGVINVVA	800
801	TIGELAQVSG LEMRKRVDEL FIIIMDQD SSLLAKRQVA LWTLGQVLASI	850
851	TGIVVPEYRK YPTLLEVLLN FLKTEQNOGT RREAJRVLGL LGALDPYKHG	900
901	YKIGTNDOSR DASVLSIES KSSQDSDYS TSEMLVAMH LPLDEFYPAV	950
951	SAVALRMRIF DOLS4HHTH VVQAITFTK SLGKCVQFL PIVMPFTFLNV	1000
1001	IYRCDAIGARE FLPQLQHMLV SFVKSIRHY MOEIVTULARE PWVWNTSIO	1050
1051	TIILLIEQIV VALGGEGFKL LPQLZIPMLR VFHMHDNSPR IVSIKLLAAI	1100
1101	QLFGANLDDOY LHLLPPPIV LFDAPEAFLP SRKAALETYD RLIESLDETT	1150
1151	YASRIIHPH YKIDQSBEP STANDTSSL VFQLGKQYQI FIPMVNKVLY	1200
1201	RHRINHORVD VLICTRIVKGY TLADEEFOPL TYQHBMLASG OGDALASCPV	1250
1251	ETGPWKKLHV STINOKANG AABRVSQDKD LEWLRRLSLE LLDKSSSPS	1300
1301	RSCHLAQAOY NPMDRDLFA AFVSCWQAY EDQDDELRIS IELALTSQDZ	1350
1351	AEVQTTLNLN AEFHENSDK PLPLRONGI VLLGERAHC RAYAKALHYR	1400
1401	ELEQKQGPT AILESLISIN NKLQPEAA GVLEYAMHNE GELETOATHY	1450
1451	EXLHNEDAL VAYDKQMDN KDDPELNLG RNCRLEALGW GQHOCCKE	1500
1501	WTLYNDETOQA KMARHAAAAG WGLGQDSNE EYTCMIPROT HDGAVYRVL	1550
1551	ALHQDLSKAQ QOCQKARQD LDAELTAMAG ESYSSRAYGA VSCBMLSELE	1600
1601	EVQYKLVPA RREIZRQINQ ELERGQCPY EDWQXILAVR SLVVSPEHM	1650
1651	RFLKLYASPL GKSRRGLALARH KTVLILQDQ PSLRQHPLP VHPQVITYA	1700
1701	MKRMWHSARK ZDAFQHMQHF VQTMQQDQAH AZATEDQGHC QELKHLMAR	1750
1751	FLKLGEQWLQ LNQINESTIP KVLYQYSAAT EHORSWYKAN HAWAVMNFEA	1800
1801	WLYYKQHNOQ RDEKKNLRLA SCANITATT ATTAAATTATT TASTEGSNSE	1850
1851	SEASESTNSP TPSPLQKQVY EDSLXILLYN TVPAVGFFR SISLSRGNNL	1900
1901	QOTLRLVTLP FDYGHNPDVW EALVEGKAI QDQTMQWIP QOLARIOTPR	1950
1951	PLVGRLQHOL TLYOQYHNPQ ALIYPLTVAS KSTTARHNA ANKILKHNCE	2000
2001	HSTNTLVQAM MVESEELIRVA IHLRNENHEG LEEASRLYFG ERNVKGKMFV	2050
2051	LEPLHMMER GPQTKLSETE NOAYGRDNLN AQEWCRKYM SGNVQLDTQ	2100
2101	WDLYYHVFRR ISKQPLOTS LEQYQSPKL LNCRQELAV PGYDPMNPRT	2150
2151	TKLILKSPPL QVYTSKQPR KTLTNGSNGH EFVFLLKGHE DLQDQERVAQ	2200
2201	LFGCLNTLLA NDPTSXKRLN SIQYRQHNSA STNSGLIOWN PHCDTLHALI	2250
2251	RDYREKKKIL NLHEDRHNQI MADPYDHLT MAMVYVFEHA VNTAGDQLA	2300
2301	KILNLKSPSS EVNFRDHTNY TRSLAVMSVY GTIQLGLORH PSNLWMLDRS	2350
2351	GKILHIDFGD CFEVANTREK FPEKFLPRLT RHLTNAMEVT GLDGMYRITC	2400
2401	HTVVEYLREH KOSVHVALEK FVYDPLLNRAK LMQNTTRQKQ BSRTRTDSYS	2450
2451	AGOSVETLDC VELGEPAKKK TGTTPESHTH SEIGDGGLVKP FALNKKAIQI	2500
2501	INRVRQKLTG RDPSHOTLD VPTQVELLIK QATSHENLQ CYIGWCPEW	2549



the FKBP12-rapamycin target less effectively than FKBP12-rapamycin itself.

A fusion protein of glutathione-S-transferase with FKBP12 (GFK) was used to identify candidates for the biologically relevant targets of FKBP12-rapamycin. MG-63 cells were lysed by detergent and complexes of GFK-rapamycin, GFK-FK506 or GFK alone were added individually to clarified lysate at a final concentration of 500 nM or 2.5 μ M (Fig. 1a). A protein of approximate relative molecular mass 220,000 ($M_r \sim 220K$) was detected in the GFK-rapamycin sample by SDS-PAGE and silver staining (Fig. 1a, lane 3). This FKBP-rapamycin-associated protein (FRAP) was not retained with GFK-FK506 or GFK alone (Fig. 1a, lanes 1 and 2). No other rapamycin-specific proteins were detected by silver staining (Fig. 1a) or by a similar